TALENs-mediated gene disruption of myostatin produces a larger phenotype of medaka with an apparently compromised immune system

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ABSTRACT

Although myostatin, a suppressor of skeletal muscle development and growth, has been well studied in mammals, its function in fish remains unclear. In this study, we used a popular genome editing tool with high efficiency and target specificity (TALENs; transcription activator-like effector nucleases) to mutate the genome sequence of myostatin (MSTN) in medaka (Oryzias latipes). After the TALEN pair targeting OlMyostatin was injected into fertilized medaka eggs, mutant G0 fish carrying different TALENs-induced frameshifts in the OlMSTN coding sequence were mated together in order to transmit the mutant sequences to the F1 generation. Two F1 mutants with frameshifted myostatin alleles were then mated to produce the F2 generation, and these F2 OlMSTN null (MSTN−/−) medaka were evaluated for growth performance. The F2 fish showed significantly increased body length and weight compared to the wild type fish at the juvenile and post-juvenile stages. At the post-juvenile stage, the average body weight of the MSTN−/− medaka was ~25% greater than the wild type. However, we also found that when the F3 generation were challenged with red spotted grouper nervous necrosis virus (RGNNV), the expression levels of the interferon-stimulated genes were lower than in the wild type, and the virus copy number was maintained at a high level. We therefore conclude that although the MSTN−/− medaka had a larger phenotype, their immune system appeared to be at least partially suppressed or undeveloped.

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1. Introduction

Myostatin (MSTN), which is also known as growth differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily. After protein secretion and proteolytic processing, a C-terminal active domain is generated and this subsequently forms an MSTN C-terminal homodimer. The MSTN C-terminal dimer then binds to membrane receptors to trigger activation of Smad2/3 [1–3]. The activation of Smad3 leads to the suppression of the expression of downstream myogenic genes [4,5], and MSTN has been shown to be a negative regulator of myogenesis [2,3].

In the MSTNs identified from mammals and fishes, the sequence of the C-terminal active peptide is conserved, with an amino acid identity of nearly 90% [6,7]. However, although a double muscle phenotype is commonly seen in MSTN null mammals, larger phenotypes are not so consistently found in MSTN-deficient higher teleost fish [8–10]. These inconsistencies arise because the MSTN gene is often duplicated (or even reduplicated) in fish but not in mammals [6]. Therefore, to investigate the role of MSTN in fish myogenesis, it is important to identify suitable fish species and a methodology which is conveniently able to suppress MSTN in these fish.

A trade-off between growth and immunity is seen in a wide range of animals from mammals through to fish and insects [11–13]. Often, however, the molecular mechanisms that underlie this trade-off remain unclear, and there is therefore a need for good
model organisms in which these mechanisms can be conveniently studied. Inhibition of the MSTN has been considerable to be tested as a potential therapy for muscle degenerative diseases like muscular dystrophy [14]. Chronic kidney disease and obesity mice increase the MSTN expression which could be related to NF-kB-dependent pathway and p38 MARK pathways [15], suggesting that MSTN expression level is probably important for a host immunity.

Medaka (Oryzias latipes), also known as Japanese rice fish, has been widely used as a lower teleost fish model to investigate gene function [16]. We selected this fish for our study because the entire medaka genome sequence is already known [17], and because it containing only a single ortholog of the mammalian MSTN [18]. Knowledge of the entire genome also makes it easy to use genome editing techniques to specifically mutate the MSTN gene.

Because genome editing with engineered nucleases (GEEN) is quick and easy compared to traditional knock-out methods, techniques such as transcription activator–like effector nucleases (TALENs) have recently been applied in many studies across a range of species [19–21]. The TALEN backbone contains the FokI nuclease fused with a DNA binding domain derived from the transcription activator–like (TAL) effector of plant pathogenic bacteria in the genus Xanthomonas [22]. The target genomic DNA binding specificity of TALENs is determined by a series of tandem repeats, called repeat variable di-residues (RVDs) [23,24]. These series of tandem repeats individually target the sense or antisense strands of the genomic sequence of interest, and after target DNA binding, the DNA double-strand break (DSB) is cleaved by a dimer of fused FokI nucleases that is attached to the TAL effectors [25]. The nonhomologous end-joining (NHEJ) repair system will subsequently be triggered, and the end result will be a disrupted gene with insertion and/or deletion (indel) mutation [26].

In this study, we successfully generated MSTN mutated medaka from the G0–F3 generations by using TALENs-mediated mutagenesis. We investigated the expression of Smad2/3-regulated downstream MRFs and the growth performance of the TALEN-mediated MSTN-mutated F2 medaka. Further, since recent studies have shown that MSTN null/silencing may alter the immune responses [15,27], we also used a virus challenge test to investigate the effect of TALENs-mediated MSTN-mutation on the expression of interferon-stimulated genes (ISGs) in MSTN-mutated (MSTN−/−) F3 medaka. To our knowledge, this is the first report on the growth and immune responses of TALENs-mediated MSTN mutant fish and their offspring.

2. Materials and methods

2.1. Design and construction of MSTN-TALENs

Medaka MSTN was identified from the Ensembl medaka genome browser (Ensembl gene No. ENSORLG00000015057). OiMSTN-TALENs were designed and constructed following the protocol as previously described by Ansai et al. [28]. Briefly, we searched for potential TALENs target sites upstream of the MSTN C-terminal mature domain using the TALE-NT 2.0 program (https://tale-nt.cacornell.edu/) [29] with the following parameters: (i) 14–17 bp for spacer length; (ii) 15–18 bp for repeat array length; (iii) only 1 in the upstream base. The TAL effector modules were assembled and cloned into the array plasmids pFUS by the Golden Gate assembly method [30]. The target sites of the MSTN-TALENs RVD modules for the left and right binding arms were NN-NH-NH-HD-NH-NN-HD-NH-NH-NH-NN-NH-NN-HD-HD-HD-HD-NH-NN-HD-HD-NH-NN-HD-NH-NH-HD-NH-NH-HD-NH-HD-NH-NH-HD, respectively. The assembled repeat arrays were then respectively cloned into the expression vectors pCS2TAL3DD and pCS2TAL3RR [31].

2.2. mRNA synthesis and microinjection of MSTN-TALENs

TALENs mRNA was synthesized using the protocols described in Ansai et al. [28]. Briefly, TALENs mRNA was synthesized in vitro from the Norl linearized TALEN expression vectors using an MMessage mMachine SP6 kit (Life Technologies). Before microinjection, the transcribed mRNAs were purified using an RNasy Mini kit (Qagen) and diluted with Yamamoto’s Ringer’s solution (0.75% NaCl, 0.02% KCl, 0.2% CaCl₂, and 0.002% NaHCO₃, pH 7.3) [32]. Finally, MSTN-TALENs mRNA pairs of the same dosage as in Ansai et al. [28] were simultaneously microinjected into Cab strain fertilized eggs at the single cell stage using the medaka microinjection method as previously described in Kinoshita et al. [33].

2.3. Mutation analysis of MSTN-TALENs mediated medaka

To observe the mutation genotypes of the TALENs-mediated MSTN-mutated medaka, genomic DNA was individually extracted from the caudal fin of adult fish by using the methods of alkaline lysis (25 mM NaOH and 0.2 mM EDTA) and neutralization (40 mM Tris–HCl, pH 8.0) as described in Ansai et al. [28]. Subsequently, the primer set OiMSTN-F1/0iMSTN-R1 (Table 1) was used to amplify the target sequence of the MSTN-TALENs. The resulting amplicons were cloned into TA cloning vector (Bioman, Taiwan) and sequenced.

2.4. Quantification of the mRNA expression of myogenic regulatory factors (MRFs) in MSTN-mutated (MSTN−/−) F2 medaka using real time PCR

To determine the gene expression of the myogenic regulatory factors, skeletal muscles were taken from four to eleven individual samples of wild type (WT; i.e. non-genome-edited Cab strain) F2 medaka and from MSTN-mutated F2 medaka at the juvenile and post-juvenile stages (2 and 4 weeks post-hatching, respectively). Total RNAs from these skeletal muscle samples were extracted by ReZol &T reagent (Protech Technology, Taiwan), and cDNAs were synthesized by Superscriptase II Reverse Transcriptase (Invitrogen) with Anchor-dTv primer. The relative expression of these MRFs (MyoD, Myf5, and Myogenin) and EF1α housekeeping gene were quantified by real-time PCR with the primer sets MyoD-Q-F/MyoD-Q-R, Myf5-Q-F/Myf5-Q-R, Myogenin-Q-F/Myogenin-Q-R, and EF-1α-Q-F/EF-1α-Q-R. The relative expressions of the MRFs were then normalized with respect to EF-1α using the $2^{ΔΔCT}$ method. Data values were proportionally adjusted to the WT expression level, which was set to 1. Statistically significant differences between WT and MSTN−/− F2 medaka were calculated by student’s t-test. Primer sequences are listed in Table 1.

2.5. Growth assessment of MSTN-mutated (MSTN−/−) F2 medaka

To observe the phenotype of MSTN-mutated (MSTN−/−) F2 medaka, the body weight and standard length were measured using a modified version of the protocol described previously by Chisada et al. [18]. In brief, thirty fertilized eggs of WT and MSTN-mutated (MSTN−/−) F2 medaka were incubated in the medaka embryo culture medium (0.0001% methylene blue, 0.1% NaCl, 0.3% KC1, 0.004% CaCl₂·2H₂O, 0.016% MgSO₄·7H₂O). After hatching, twenty larvae were randomly chosen from each group, and maintained in 10 L plastic rearing containers under a 14/10-h day/night cycle at 26 °C. During the measurement of body weight and standard length, fish were anesthetized in 0.003% eugenol (Sigma–Aldrich, Inc., MO, USA). The body weight and standard length were measured in 10 individuals from each group at 2, 3, 4, and 5 weeks post-hatching. Because cumulative mortality increased over time, in week 7, only 7 and 9 WT and MSTN−/− individuals were
measured, respectively. In week 8, only 4 and 3 surviving individuals were measured.

2.6. Red spotted grouper nervous necrosis virus (RGNNV) bath challenge and the expression of interferon-stimulated genes (ISGs) in MSTN-mutated (MSTN<sup>−/−</sup>) F3 medaka

Medaka larvae were challenged by immersion following the method described by Furusawa et al. [34]. Briefly, one week after hatching, WT (i.e., non-genome-edited strain Cab F3) and MSTN<sup>−/−</sup> F3 larvae were transferred to a tank containing 50 ml ultraviolet-treated water with 10<sup>5</sup> TCID<sub>50</sub> RGNNV/L and 5 μg/ml kanamycin. (MSTN<sup>−/−</sup> F3 larvae were used in this experiment because MSTN<sup>−/−</sup> F2 larvae were not available in sufficient numbers.) Control WT and MSTN<sup>−/−</sup> F3 larvae were exposed to the same quantity of PBS vehicle only and 5 μg/ml kanamycin under the same conditions. Cumulative mortality was recorded daily in one group of challenged WT and in one group of challenged MSTN<sup>−/−</sup> medaka. In four other groups (i.e., one each of WT-PBS, WT-NNV, MSTN<sup>−/−</sup>-PBS, and MSTN<sup>−/−</sup>-NNV), to monitor the gene expressions of interferon-stimulated genes (ISGs) and RGNNV RNA2, total RNAs were extracted from three whole larvae at 1, 3 and 6 days post PBS- and RGNNV-treatment by the extraction method described above and then subjected to cDNA synthesis using Superscriptase II Reverse Transcriptase (Invitrogen) with Random hexamer primer (Table 1). The relative expressions of four ISGs (ISG15, ISG56, Giga1, and Mxa) were quantified by real-time PCR with the primer sets ISG15-Q-F/ISG15-Q-R, ISG56-Q-F/ISG56-Q-R, Giga1-Q-F/Giga1-Q-R, and Mxa-Q-F/Mxa-Q-R (Table 1). Real-time PCR was also used to quantify the relative expression of RGNNV using the RNA2<sub>qPCR</sub>F/RNA2<sub>qPCR</sub>R primer set (Table 1). Although the NNV we used in this study was collected from orange spotted grouper [35], this NNV strain is the red spotted grouper NNV. The NNV RNA2<sub>qPCR</sub>F/RNA2<sub>qPCR</sub>R specific primer set was normalized with respect to EF-1α using the 2<sup>−ΔΔCT</sup> method as described above. The data values of the ISGs were proportionally adjusted relative to the WT-PBS expression level, which was set to 1. For the ISGs, statistically significant differences between each group were calculated by Tukey’s multiple-comparison test (SPSS computer software). For NNV RNA2 expression, a student’s t-test was used to test for statistically significant differences.

3. Results

3.1. OlMSTN-TALENs design and construction

The MSTN genes are highly conserved across species. As with both mammals and other teleosts, the medaka MSTN gene is organized into three exons and two introns (Ensembl gene no. ENSORLG0000015057; Fig. 1A). The medaka MSTN gene encodes 377 amino acids that span three conserved domains: a signal peptide in exon 1, an N-terminal pro-peptide in exon 2 and an

![Image](305x153 to 549x303)

**Table 1**

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<sup>a</sup> AC: accession number.

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![Image](Fig. 1. Genomic structure of OlMSTN and the target location of OlMSTN-TALENs. (A) Schematic representation of the OlMSTN gene (Ensembl gene No. ENSORLG0000015057). The OlMSTN gene contains three exons (white boxes) and spans approximately 3.5 kb. The active C-terminal domain (gray box) is in the third exon of the OlMSTN gene. (B) Design of the OlMSTN-TALENs. Our OlMSTN-TALENs platform targeted the second exon. The left and right recognition sequences are indicated by green and orange boxes, respectively. The DD and RR heterodimeric FokI domains are respectively fused with the left and right TALENs effectors (OlMSTN-L and OlMSTN-R). The four RVD-nucleotide pairs are also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
active C-terminal domain in exon 3. However, unlike in other teleosts, whose MSTN genes are duplicated, there is only one orthologous MSTN gene in the medaka genome [18,36]. To prevent expression of the MSTN active C-terminal domain encoded by exon 3, we used TAL Effector-Nucleotide Targeter 2.0 [29] to design a TALENs that targeted the 5’ end of exon 2 of the medaka MSTN genome. The left and right TAL effectors recognize sequences that are separated by a 16 bp spacer, and they each contain 15 RVDs that are fused with the DD or RR heterodimeric FokI domain, respectively (Fig. 1B).

3.2. Obtaining the TALENs-mediated F1 medaka mutants and the F2/F3 MSTN-mutated (MSTN<sup>−/−</sup>) medaka

To obtain the parent generation of MSTN-mutated medaka, G0, a pair of OlMSTN-TALENs (200 ng/μl) was injected into fertilized medaka eggs. After hatching, the fish were screened for TALENs-mediated MSTN mutation by extracting genomic DNA and amplifying the 175-bp MSTN-TALENs target site by PCR. The amplicons were subcloned and sequenced. Consequently, four of the MSTN-TALENs-mediated mutant G0 medaka were raised to sexual maturity. These fish were crossed with each other, and their offspring were then similarly screened for TALENs-mediated MSTN mutated medaka (F1 progeny). Thirteen mutated progeny were identified and the mutations of their MSTN genes were categorized as frame-shifted mutations (3F, 8F and 11M), in-frame mutations (1M, 2F, 5M, 21M and 22M), or a combination of both in-frame and frame-shifted mutation (15M, 17M, 18F, 19M and 23M) (Fig. S1). These results suggested that the TALENs-mediated MSTN mutations could be transmitted to the next generation.

Subsequently, because the TALENs-mediated MSTN-mutated F1 female medaka 8F and the TALENs-mediated MSTN-mutated F1 male medaka 11M both possessed the same mutation pattern in their genome, these two mutants were selected and mated to generate an F2 generation of MSTN mutant medaka (MSTN<sup>−/−</sup> medaka) (Fig. 2). All six of the randomly tested F2 progeny had one of the two frame-shifted genotypes shown in Fig. 2. One genotype was a homozygous frame-shift mutation with the same 4-bp deletion and 6-bp replacement, while the other was a heterozygous mutation consisting of the 4-bp deletion and 6-bp replacement plus a 16-bp deletion (Fig. 2; Table S1). For the challenge experiment, pairs of the heterozygous F2 mutants were crossed to produce F3 generation MSTN<sup>−/−</sup> mutants. As before, we checked the genomic DNA of ten randomly selected F3 mutants and found that they all had one of the two frame-shifted genotypes shown in Fig. 2. These results show that MSTN-TALENs induced frame-shift mutations in exon 2 of the medaka MSTN genome, and that these mutations were transmitted to the next generation. Since the MSTN gene was completely disrupted in the F2 genotypes, we inferred that the MSTN protein was no longer functional.

3.3. Effect of TALENs-mediated MSTN mutation on the expression of MRFs in mutated F2 medaka

To investigate the effect of TALENs-mediated MSTN frame-shifted mutations on the expression of myogenic regulator factors (MRFs), we extracted the mRNA from the skeletal muscle of juvenile and post-juvenile wild type (WT) medaka and the F2 generation of homozygous MSTN mutant medaka (MSTN<sup>−/−</sup> medaka), and used real-time PCR to detect the expression of three MRFs: MyoD, Myf5, and Myogenin.
Myf5 and Myogenin.

As shown in Fig. 3A, at the juvenile stage, only MyoD gene expression was significantly increased in the MSTN−/− F2 fish relative to the wild type. At the post-juvenile stage, in the MSTN−/− F2 fish, the expression levels of MyoD, Myf5 and Myogenin were significantly increased by 19%, 31% and 56%, respectively (Fig. 3B). These results indicate that TALENs-mediated MSTN frame-shifted mutation induced the expression of MyoD at the juvenile stage and subsequently also up-regulated the expression of Myf5 and Myogenin at the post-juvenile stage.

3.4. Effect of TALENs-mediated MSTN mutation on growth performance of mutated F2 medaka

To monitor growth performance, we tracked the standard length and body weight of wild type and MSTN−/− F2 fish from the juvenile through to adult stages (i.e. from 2 to 8 weeks after hatching). Both of these values were significantly higher in MSTN−/− F2 fish than in the wild type fish at each tested time point except for week 7 (Fig. 4A and B). At 5 weeks after hatching, the increase in body length and body weight was 15.3% and 80%, respectively, after hatching), the relative length and weight showed an increase from 2 to 8 weeks after hatching), the relative length and weight showed an increase of 13.4% and 24.5%, respectively (Fig. 4A and B). We also note that in terms of fish morphology, except for the increased length and weight, there were no observable defects or deformities in MSTN−/− F2 fish (Fig. 4C). These results suggest that TALENs-mediated MSTN frame-shifted mutated (MSTN−/−) medaka present a larger phenotype than the wild type fish.

3.5. Effect of TALENs-mediated MSTN mutation on the response to virus challenge in mutated F3 medaka

As the first step, to investigate whether there is a trade-off between growth and immune function in the TALENs-mediated MSTN mutated medaka, F3 MSTN−/− fish were challenged with RGNNV by immersion. Larval medaka were used for this challenge because at one week old, these fish are highly susceptible to NNV. After challenge, the larvae were collected at different time points and subjected to total RNA extraction and cDNA synthesis. The virus susceptibility and the expression of interferon-stimulated genes (ISGs) were then measured using real-time PCR at various times post-challenge.

To assess the virus susceptibility of the MSTN−/− fish, the expression of NNV RNA2 was used as a proxy to indicate the RGNNV copy number and thus the NNV infection state. Fig. 5A shows that, although initially there was no significant difference in the expression of RGNNV RNA2 between the WT and MSTN−/− fish, RNA2 expression was reduced much faster in the WT fish. From this we infer that the virus was cleared more slowly in the MSTN−/− fish. Nevertheless, cumulative mortality was similar in both groups, increasing rapidly after 2 dpi and reaching 100% mortality at 7 dpi (Fig. 5B).

Next, to investigate the anti-viral immune response, the expression of several interferon-stimulated genes (ISGs), including G1m, MxA, ISG56 and ISG15, were observed at various times in the wild type and MSTN−/− fish. As shown in Fig. 6, without RGNNV challenge (PBS treated group), the expression level of ISGs in the MSTN−/− fish was the same as that in the wild type. After RGNNV challenge, in the wild type fish, all of the ISGs except ISG15 were significantly upregulated at one or two of the three time points. By contrast, none of the ISGs were significantly induced in the MSTN−/− fish after RGNNV challenge.

4. Discussion

In this study, we used TALENs in a genome-editing strategy to successfully generate MSTN mutated (MSTN−/−) medaka. Although TALENs-mediated MSTN gene editing has been applied in mammals such as mice, cattle and sheep [37,38], this is the first time that an MSTN-TALENs has been designed for medaka. Like all of the mammalian TALENs-mediated MSTN gene editing platforms, we also targeted a site that is upstream of the active domain in the MSTN C-terminal. The heritability of TALEN-mediated MSTN mutation in medaka was also confirmed (Fig. 2), indicating that TALENs is a tool that can easily be used to establish mutant lines that are stable.

MSTN is already known to be a specific negative regulator of muscle growth [2]. When MSTN binds to type II serine/threonine kinase receptor to activate the Smad2/3 pathway, the expression of the MRfs is suppressed [39]. Conversely, when MSTN is inhibited or loses its functionality, the activation of Smads is reduced and this leads to an increase in MRfs expression [18,40]. Since the effect of MSTN on Smad signaling is observed in both mammals and fishes, this signaling transduction pathway is evidently highly conserved. Our results also show that, in MSTN−/− medaka, MRfs were significantly increased at the post-juvenile stage, compared to the wild type (Fig. 3). Similar results were also found in mstnC315Y medaka, which are MSTN deficient fish in which an MSTN C315Y-substitution mutation was produced by EMU-mutagenesis [18]. Although Chisada et al. [18] found no significant induction of
Fig. 4. Growth evaluation of MSTN<sup>−/−</sup> medaka. (A) Body weight and (B) standard length of wild type (WT) and MSTN<sup>−/−</sup> (F2) medaka. Fish were sampled at 2, 3, 4, 5, 7, 8 weeks post-hatching. From 2 to 7 weeks post-hatching, values represent the mean ± SD from seven to ten individual samples. At 8 weeks post-hatching, three or four individual samples were measured. Asterisks indicate a significant difference (*p < 0.05, **p < 0.01 and ***p < 0.001) between WT and MSTN<sup>−/−</sup> medaka. (C) Morphology in the juvenile and adult stages of wild type (WT) and MSTN<sup>−/−</sup> (F2) medaka.

Fig. 5. Expression of NNV RNA2 and cumulative mortality in MSTN<sup>−/−</sup> medaka after RGNNV challenge. (A) Real-time PCR analysis was used to investigate the expression of RGNNV RNA2 in WT and MSTN<sup>−/−</sup> medaka (F3) after immersion challenge with RGNNV. The RGNNV RNA2 was used as an indicator of the RGNNV infection status. The expression level of RGNNV RNA2 was normalized against EF-1α mRNA levels. Each bar represents the mean ± SD from five individual samples. Asterisks indicate a significant difference (*p < 0.05, **p < 0.01 and ***p < 0.001) between WT and MSTN<sup>−/−</sup> medaka. (B) Cumulative mortalities in the WT and MSTN<sup>−/−</sup> medaka.
Fig. 6. Expression of interferon-stimulated genes (ISGs) in MSTN⁻/⁻ medaka after RGNNV infection. Real-time PCR analysis was used to investigate the expression of the interferon-stimulated genes (ISGs) (A) OlIGg1, (B) OlMxA, (C) OlISG56, (D) OlISG15 in WT and MSTN⁻/⁻ medaka (F3) after immersion challenge with RGNNV. The expression levels of the ISGs were normalized against EF-1α mRNA levels, and the levels of the WT PBS group were set to 1. Each bar represents the mean ± SD from five individual samples. Different letters indicate significant differences between treatments and/or between the wild type and MSTN⁻/⁻ fish at the same time point.
several important MRFs (Myf5, MyoD and Myogenin) in mstnC315Y at the juvenile stage (2 weeks post-hatching), these MRFs were subsequently maintained at a high level and this continued through to the early adult stage (8 weeks post-hatching). At the late adult stage (16 weeks post-hatching), only Myogenin was significantly up-regulated [18]. On the other hand, whereas Chisada et al. [18] showed that Smads activation was suppressed in mstnC315Y medaka, even when we used the same commercial phospho-Smad2 antibody, we were unfortunately unable to produce sufficiently clear signals to show whether Smads was activated or not in our MSTN mutants (data not shown). Nevertheless, the increase in MRFs (Fig. 3) was enough to suggest that MSTN function was disrupted in our TALENs-mediated MSTN mutated medaka.

Unlike in mammals, the MSTN phenotype in fish has not yet been well defined. For example, while a 40% increase of body weight was observed in RNAi-mediated MSTN silenced zebrafish [8], overexpression of a dominant-negative MSTN had almost no effect and only produced slight increase in the body weight of both zebrafish and medaka [10,36]. In the present study, our TALENs-mediated MSTN mutated medaka showed a significantly improved growth rate in terms of body weight and length (Fig. 4), and the observed difference was significant at 8 weeks after hatching. In contrast, M. Schwartz et al. [15] showed a 13.4% and 24.5% increase in body length and body weight, respectively (Fig. 4A and B). The growth performance of TALENs-mediated MSTN mutated medaka also seems to be at least as good as the mstnC315Y mutants. Taken together, these results suggest that complete disruption of MSTN on the genomic level leads to an enhancement of the medaka growth rate.

In both terrestrial and aquatic farm animals grown as food for humans, such as cows, pigs, poultry and trout, loss of myostatin function enhances growth and is generally thought of as beneficial [9,41–43]. There is no evidence, either in previous reports or in our present study, that “myostatin blockade” has any negative effect on animal survival under normal conditions. However, for an animal, both fast-growth and maintenance of an effective immunity are costly endeavors, and there is a trade-off between these fitness-enhancing traits [13,44]. To give some examples: in poultry, selected lines with accelerated growth showed a significant reduction in their the immune response to a variety of immune challenges [44] while Foote et al. [45] reported that, although a high growth rate of bull calves was achieved by a nutrient replacer, the calves showed a decrease in immune cell viability.

To date, most MSTN research has focused on its inhibitory effect on myogenesis [3,46], and except for the immune side-effects triggered by some MSTN gene delivery platform [47,48], very few studies have explicitly investigated the immune responses in MSTN null/silenced animals. One such study was conducted by Lyons et al. [27], who found that obesity-resistant SWR mice not only had consistently lower levels of myostatin in the spleen than susceptible mice, they also had lower expression of proinflammatory cytokines, such as IFNγ, IL-1β and IL-17. Zhang et al. [15] similarly found that in mice with chronic kidney disease, the pharmacological inhibition of myostatin led to suppression of circulating inflammatory cytokines. Our present results likewise showed that TALENs-mediated MSTN mutated medaka with a larger phenotype failed to induce antiviral immune genes against virus infection. We found that in wild type medaka, most ISGs were significantly induced after challenge with RGNNV (Fig. 6), and the virus copy number decreased more or less in parallel (Fig. 5A). By contrast, the TALENs-mediated MSTN mutated medaka failed to induce an inflammation response and showed a low efficiency in eliminating the virus (Figs. 5A and 6). These results suggested that, at least in medaka, myostatin may somehow serve as a modulator for the innate anti-viral immune system. We note, however, that MSTN may not always be involved in a straightforward trade-off between growth and immune function, as shown in a study by Wick et al. [49]. When Wick et al. treated mouse C2C12 myoblasts with exogenous MSTN to investigate MSTN-mediated mechanism of myogenesis, their DNA microarray results revealed not only the expected disruption of cytoskeletal and muscle-specific protein expression but also a decrease in a large number of genes involved in the inflammation mediated by the chemokine and cytokine signaling pathways. Clearly, even in terrestrial animals, the role of myostatin in the immune system is still not fully understood. We would therefore advise further study of the teleost immune response before MSTN-deficient fish are adopted by the industry.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.11.016.

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